DATA EVALUATION RECORD
AQUATIC PLANT TOXICITY USING *LEMNA* SPP.
GUIDELINE OPPTS 850.4400

1. **CHEMICAL:** Bardac 22C50 (Carboquat) **PC Code No.:** 069208

2. **TEST MATERIAL:** Carboquat **Purity:** 49.85%
   Batch No.: 5628-137

3. **CITATION**

Author:	Debbie Desjardins, B.S. Jon A. MacGregor, B.S. Henry O. Krueger, Ph.D
Title:	A 7-Day Toxicity Test of Bardac 22C50 with Duckweed (*Lemna gibba* G3)
Study Completion Date:	May 28, 2004
Laboratory:	Wildlife International, Ltd. 8598 Commerce Drive Easton, Maryland 21601
Sponsor:	Lonza Inc. 17-17 Route 208 Fair Lawn, New Jersey 07410
Laboratory Report ID:	289A-159
DP Barcode:	D305528
MRID No.:	462958-02

4. **REVIEWED BY:** Kathryn V. Montague, Biologist US EPA/AD/RASSB

   Signature: **Date:**

5. **APPROVED BY:** Siroos Mostaghimi, Team Leader US EPA/AD/RASSB

   Signature: **Date:**

6. **STUDY PARAMETERS**

   Study Type: Static
   Definitive Study Duration: 7 Days

7. CONCLUSIONS

Verified Results Synopsis:

Frond Growth:
EC50: 118.78 Φg a.i./L
95% Confidence Interval: 58.9 and 471.0 Φg a.i./L
Slope = 2.11

Growth rate:
EC50: 199.1 Φg a.i./L
95% Confidence Interval: 118.3 and 650.7 Φg a.i./L

NOAEC: 9.1 Φg a.i./L (based on frond growth)

8. ADEQUACY OF THE STUDY

A. Classification: Acceptable (Core)

B. Rationale: No significant deviations from Guideline requirements.

C. **Repairability:** N/A

9. GUIDELINE DEVIATIONS: The following guideline deviations were based on EPA OPPTS Guideline 850.4400 (EPA 712-C-96-156):

- $ The guideline states that pH should be measured prior to testing and on days 3, 5, and 7. The pH was not measured on days 3 or 5.
- $ Raw data not provided.
- $ The colonies were not transferred to replacement test solutions on days 3 and 5 to prevent nutrient limitation or depletion.
- $ EC5's, EC90's and LOAEC=s were not calculated or plotted. No goodness-of-fit was determined.
- $ The range of chemical concentrations selected for testing resulted in the highest concentration affecting only 74% of the fronds by day 7. The guideline requires 90% of the fronds to be affected. However, the EC50 was bracketed by the highest two test concentrations.
- $ Concentration response curves were not plotted for total frond number, growth rate (as number of fronds per day) and mortality (percentage of dead fronds to total number of fronds).
- $ A concentration response curve for mean frond number was plotted but 95 percent

confidence limits were not delineated.

$ The means for growth rate and percent frond mortality were not plotted. Standard deviations were not reported for any calculations in the Study Report.

$ The following information was not reported:

- If stock culture grown from a single isolated plant was used to inoculate all the flasks in a given test.
- The methods and results of the range-finding test
- If the laboratory runs positive controls with zinc chloride as a reference chemical periodically to ensure that the test organism is responding to a known chemical in an expected manner
- If the analytical method was validated.

10. SUBMISSION PURPOSE: Registration

11. MATERIALS AND METHODS

A. Test Organisms:

Guideline Criteria	Reported Information
Species:	
$ *L. gibba* G3 and *L. minor*	$ *L. gibba* G3
$ Cultures obtained from laboratory or commercial sources.	$ The original duckweed cultures were obtained from the United States Department of Agriculture (p.11)
$ Stock culture grown from a single isolated plant should be used to inoculate all the flasks in a given test.	$ Not Reported
$ Axenic stock cultures should be grown in an aquarium for 2 weeks prior to use.	$ Cultures had been actively growing in 20X AAP culture medium for at least two weeks prior to test initiation. (p.11)
Plants:	
$ Three to five plants consisting of three to four fronds each per replicate.	$ Five plants totaling 15 fronds were added to each replicate test chamber. (p.10)

B. Test System

Guideline Criteria	Reported Information
Nutrient Media:	
$ M-Hoagland=s or 20X-AAP nutrient media	$ 20X-AAP nutrient medium (p.11)
$ Medium should be prepared prior to each transfer of *Lemna* cultures and for preparation of new test solutions during the course of the test.	$ Medium was not freshly prepared throughout the test as *Lemna* cultures were not transferred as required.
$ If M-Hoagland=s medium is used pH is adjusted to between 4.8 and 5.2 by addition of 0.1N or 1 N NaOH.	
$ If 20X-AAP medium is used pH is adjusted to 7.5 ∀ 0.1 with 0.1 N NaOH or HCL.	$ The pH was adjusted to 7.6 using 10% HCl. (p.11)
Test Container:	
$ At least three replicate containers should be used for each concentration, each containing 150 mL of test solution, or enough test solution to result in a volume-to-vessel size ratio of 2:5.	$ Three replicate containers with a volume-to-vessel ratio size of 2:5 (250-mL beakers with 100 mL of test solution). (p.12)
$ Test containers may be 250-mL glass beakers or Erlenmeyer flasks, large enough to hold 150 mL of test solution and *Lemna* colonies without crowding for the duration of the test.	$ 250-mL glass beakers (p.12)
$ The same number of replicates should be used for each test concentration and control.	$ Yes, three replicate test chambers were maintained in each treatment and control group. (p.10)
$ Test containers should be randomly placed in the environmental chamber.	$ Test containers were indiscriminately positioned daily in the environmental chamber. (p.12)
Test Apparatus:	
$ Controlled environment growth chamber or enclosed area capable of maintaining the specified number of growth chambers and test parameters required	$ Temperature controlled environmental chamber (p.12)
$ All glassware and equipment should be cleaned following good laboratory	$ The Study Report states that the test chambers were sterile but no further

Guideline Criteria	Reported Information
practice. Nytex screen or inoculating loops used for transferring the *Lemna* should be disposed of after use or thoroughly cleaned and sterilized before reuse.	information was provided regarding whether good laboratory practice was applied. (p.12)
Temperature:	
$ Environmental chamber maintained at 25 ∀ 2EC	$ Yes (p.12). Temperature measured twice daily using a hand-held liquid-in-glass thermometer was 23.7 to 24.4EC. The minimum an maximum temperature measured continuously ranged from 24 to 26EC. (P.16)
pH:	
$ If M-Hoagland=s medium is used pH is adjusted to between 4.8 and 5.2	
$ If 20X-AAP medium is used pH is adjusted to 7.5 ∀ 0.1	$ pH adjusted to 7.6 (p.11)
$ Test solution pH may vary from the nutrient medium after addition of the test chemical and/or carrier. Changes should be recorded but not adjusted.	$ No changes in pH were adjusted after the addition of the test chemical.
$ Report pH of the test chemical in the test solutions prior to use and discarding on days 3, 5 and 7.	$ pH was measured on day 0 and day 7. Only one test solution was used as no transfers of colonies were made. (p.12)
Photoperiod and Light Intensity:	
$ Continuous warm-white fluorescent lighting should be used to provide a light intensity in the range of 4,200 and 6,700 lux.	$ Continuous warm-white fluorescent lighting at an intensity of 5000 ∀ 750 lux (p.12)
$ Light intensity at each position in the incubation area should be measured and should not differ by more than 15 percent of selected light intensity.	$ Light intensity was measured at 5 locations surrounding the test chambers, and ranged from 4560 to 5240 lux. (p.16)
Transfer of Colonies:	
$ The colonies should be transferred to test solution on day 0, and to replacement solutions on days 3 and 5	$ The colonies were transferred to test solution on Day 0, but the test solution was not replaced during the 7 Day

Guideline Criteria	Reported Information
(to prevent nutrient limitation or depletion). $ No more than 20 percent of the test substance should be lost by volatilization (or other processes) between replacements. $ Transfer should be done in a clean, draft-free area as quickly as possible to minimize contamination of the colonies.	study (no renewal of test solution)
Observation of Colonies: $ Observation of frond numbers and appearance should be made of the colonies on day 0, 3, 5 and 7.	$ Observations of duckweed fronds were conducted on days 0, 3, 5, and 7 (p.14)
Preparation of Stock Solutions or Growth Media $ Stock solutions or growth media should be prepared just prior to use and diluted with water of high quality such as glass-distilled, deionized water, or ASTM Type I to obtain the test solutions $ pH of test solutions should be measured prior to and after use. $ Stock solutions of substances with low aqueous solubility may be prepared by use of organic solvents	$ Stock nutrient solutions and test medium were prepared using purified Wildlife International, Ltd. well water. (p.11) $ As colonies were not transferred throughout test, pH was only measured in the initial solution. $ No solvent required.
Solvents $ When solvent or carrier used, second set of controls should be prepared with highest concentration of substance $ Concentration should not exceed 0.5 mg/L	$ No solvent used.

C. Test Design

Guideline Criteria	Reported Information
Replacement of Nutrient Media: $ Replace nutrient media on day 3 or 5, or as needed to prevent nutrient limitation or depletion of test chemical. $ In 14 day test renewal may be necessary every 3 to 5 days.	$ Test solution renewals were not performed.
Doses/Dose Range: $ At least five concentrations of chemical, exclusive of controls, in a geometric series in which the ratio is between 1.5 and 2.0 (e.g., 2, 4, 8, 16, 32, 64 mg/L). $ The concentration range should be selected to define the concentration response curve between EC5 and EC90. $ The range of chemical concentrations should result in the highest concentration affecting at least 90 percent of the fronds and lowest concentration affecting no more than 5 percent of fronds compared with controls. Or, test concentrations should bracket the expected EC50 value.	$ Five concentrations of chemical with a ratio of 2 (nominal concentrations of 2.1, 4.7, 10, 23, 51, 113, and 250 Φg a.i./L) $ Concentration range did not define the concentration response curve between EC5 and EC90. $ The highest concentration affected only 74% of the fronds by day 7 in terms of mean frond numbers; however, the EC50 was bracketed by the highest two test concentrations. (p.24)
Preliminary (Range-Finding) Test: $ Perform range-finding test to establish whether a definitive test is necessary and to determine the concentrations for the definitive test. $ Expose *Lemna* to chemical concentration series (e.g., 0.1, 1.0, 10, 100, 1,000 mg/L) plus controls. $ Minimum of three replicates of 3 to 5 plants consisting of three to four fronds each should be added to each	$ A range-finding test was conducted but information regarding this test was not provided. (p.10)

Guideline Criteria	Reported Information
test chamber. $ Select plants of similar size and the number of plants and number of fronds should be identical or near identical as possible in each test chamber. $ At least 12, but no more than 16 fronds, per test chamber recommended. $ Plants exposed to equal volumes of each chemical concentration for 7 days. $ The highest test concentration should be at least 1,000 mg/L (except for pesticide testing under FIFRA). $ If range-finding test showed that the highest concentration of chemical tested (not less than 1,000 mg/L or the maximum pesticide label application rate) had no effect on *Lemna*, report the results and measured concentrations and a statement that the chemical is not phytotoxic. $ If range-finding test showed greater than 50 percent effect with a test concentration below the analytical detection limit, report the results and a statement that the chemical is phytotoxic below the analytical detection limit.	
Controls: $ Controls consist of same nutrient medium, number of fronds, environmental conditions, and procedures as the test containers except that none of the chemical is added. $ If a solvent or carrier is used to dissolve or suspend the test chemical, additional controls containing the solvent or carrier should be included.	$ Yes $ No solvent used

Guideline Criteria	Reported Information
$ The upper limit of the carrier volume is 0.5 mL/L and same amount of carrier should be added to each test concentration. $ Positive controls using zinc chloride should be run periodically.	$ Not Reported
Replicates Per Dose: $ For each concentration and control at least three replicate containers should be used. $ Three to five plants consisting of three to four fronds each should be used. $ Fewer replicates, each containing a greater number of colonies, may be used. But the test containers and solution volumes will have to be adjusted accordingly.	$ Three replicates (p.11) $ Five plants with a total of 15 fronds were added to each replicate test chamber (p.11)
Duration of Test: $ 7-days	$ 7-days
Observations: $ Colonies should be inspected for changes in frond number and appearance at the beginning of day 0, days 3 and 5, and at the end of the exposure (day 7). $ On day 7 count the number of living and/or dead fronds.	$ Colonies were inspected on days 0, 3, 5, and 7 for growth, total number of plants per replicate, and effects such as necrosis, chlorosis, dead, small, curled fronds, and root destruction of duckweed colonies. (p.15) $ Yes, determined through direct counts at exposure termination. (p.15)

12. REPORTED RESULTS

Guideline Criteria	Reported Information
$ Quality assurance and GLP compliance statements included in report?	$ Yes
$ Concentration response curves should be plotted for total frond number, growth rate (as number of fronds per day) and mortality (percentage of dead fronds to	$ Concentration response curve only plotted for mean frond number (p.27)

Guideline Criteria	Reported Information
total number of fronds).	
$ Means and standard deviations for frond number, growth rate, and percent frond mortality calculated and plotted for each treatment and control.	$ Mean frond number, growth rate, and percent frond mortality was calculated for each treatment and control. Mean frond number was plotted. No standard deviations were provided for any of these parameters. (p.23, p.24, and p.25)
$ Concentration response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and EC5s, EC50s, and EC90s, LOECs, and NOECs identified.	$ EC50's and a 7-Day NOAEC based on frond growth and growth rate determined. A concentration response curve for mean frond number was plotted but 95 percent confidence limits were not delineated.
$ Report any change in frond development of appearance such as increase in number (a frond is counted regardless of size as long as it is visible adjacent to the parent frond), decrease in size, necrosis, chlorosis, etc. Also report any additional observations such as sedimentation of test solution, sinking of fronds, or other abnormalities.	$ Documentation was provided on duckweed colony observations for chlorosis, necrosis, dead, small, curled fronds, root destruction, and any other abnormalities in frond or plant appearance. (p.15 and 23)

Method Validation

The Study Report states that the method used for the analysis of Bardac 22C50 in 20X AAP medium samples was based upon methodology developed by Wildlife International, Ltd. However, the study did not report any results of a method validation study, nor did it mention that a method validation study was performed prior to initiation of this study.

Observations:

Growth, defined as an increase in the total number of fronds in each replicate test chamber, was determined through direct counts on Days 0, 3, 5 and 7 of the test. In addition, the total number of duckweed plants in each replicate test chamber was determined at test termination. Observations of effects such as chlorosis, necrosis, dead fronds, root destruction and break-up of duckweed colonies were performed on Days 3, 5 and 7 of the test. In addition, any other abnormalities in frond or plant appearance were also documented.

Nominal concentrations selected for use in this study were 2.1, 4.7, 10, 23, 51, 113, and 250 Φg a.i./L. Samples collected at the beginning of the test had measured concentrations that ranged from 91% to 181% of nominal concentrations. The concentrations of the samples collected at test termination ranged from 4.8% to 36% of nominal concentrations. Due to the decrease in measured test concentrations by Day 7, the results of the study were based on the Day 0 measured test concentrations of 3.8, 4.8, 9.1, 23, 53, 117, and 249 Φg a.i./L.

Duckweed plants in each negative control replicate appeared healthy, with the exception of minor chlorosis (<1%), and exhibited normal growth throughout the test. Percent inhibition of frond growth (based on mean number of fronds) in the 3.8, 4.8, 9.1, 23, 53, 117, and 249 Φg a.i./L treatment groups at exposure termination was 2.0, -1.5, 0.43, 6.3, 13, 60, and 74%, respectively. Percent inhibition of growth rate in each of the treatment groups at exposure termination was 0.83, -0.65, 0.19, 2.8, 6.0, 39, and 58%, respectively. Treatment related effects for both frond growth and growth rate were apparent in the three highest concentrations. These included chlorotic and necrotic fronds. On Day 3, chlorotic fronds were noted at the 23 Φg a.i./L and higher treatment levels. By day 7, chlorotic fronds were observed at all treatment levels. The percentage of chlorotic fronds noted at 53, 117, and 249 Φg a.i./L on days 3 through 5 ranged from 1.6% to 1.9%, 7.9% to 15%, and 15% to 34%, respectively. Necrotic fronds were only observed at the two highest treatment levels. Necrosis on days 3 through 5 was observed in 7.8% to 18% of fronds at 117 Φg a.i./L and in 16% to 26% of fronds at 249 Φg a.i./L.

Frond Numbers, Growth Rate, and Percents of Inhibition at Test Termination

Day 0 Measured Concentration (Φg ai/L)	Day 7 Frond Number			Mean Day 7 Frond Number	Frond Number % Inhibition	Mean Growth Rate	Growth Rate % Inhibition
	Rep A	Rep B	Rep C				
Control	151	160	149	153	---	0.332	--
3.8	149	152	150	150	2.0	0.329	0.83
4.8	159	149	159	156	-1.5	0.334	-0.65
9.1	148	160	150	153	0.43	0.331	0.19
23	140	141	150	144	6.3	0.323	2.8
53	140	130	130	133*	13	0.312*	6.0
117	54	66	65	62*	60	0.201*	39
249	37	41	43	40*	74	0.141*	58

* Statistically significant difference on day 7 frond growth (number) or growth rate ($p<0.05$) from the control replicates using Dunnett=s test.

Day 7 Mean Percentage of Fronds Observed Dead, Chlorotic, or Necrotic Per Treatment

Day 0 Measured Concentration (Φg ai/L)	N (mean number fronds)	Mean Percentage		
		Dead	Chlorotic	Necrotic
Control	153	0	0.88	0
3.8	150	0	0.89	0
4.8	156	0	0.64	0
9.1	153	0	1.1	0
23	144	0	0.95	0
53	133	0	1.7	0
117	62	2.2	11	7.8
249	40	15	15	26

Statistical Results:

Frond Growth*:
EC50: 104 Φg a.i./L
95% Confidence Interval: 92 and 111 Φg a.i./L
NOAEC: 23 Φg a.i./L

Growth rate:
EC50: 194 Φg a.i./L
95% Confidence Interval: 143 and 229 Φg a.i./L
NOAEC: 23 Φg a.i./L

* Frond growth is the most sensitive endpoint.

Statistical Method: The Day 7 EC_{50} values were determined using linear interpolation with treatment response (frond number and growth rate) and exposure concentration data. The NOAEC was determined using analysis of variance (ANOVA) and Dunnett=s t-test after passing for normality and homogeneity of variances (p = 0.05) using the Shapiro-Wilks= and Bartlett=s tests, respectively.

13. VERIFICATION OF STATISTICAL RESULTS

Statistical results were verified using ANOVA and Dunnett's and William's Tests (TOXSTAT). The Dunnett's Test results were in agreement with the reported results; William's Test found the 7 day frond count at 23 Φg a.i./L to be significantly reduced compared to the control, making the NOEC for frond count 9.1 Φg a.i./L. Linear regression (TOXANAL) was used to verify the EC50 determination. The EC50 for frond growth was determined to be 118.78 (58.9 – 471.0) using the probit method. The slope of the dose-response curve was 2.11. This is somewhat lower than the EC50 for frond growth reported in the study .The results for growth rate were EC50 = 199.1 Φg a.i./L (95% confidence limits 118.3 and 650.7), slope = 1.9, which are similar to the reported results.

14. REVIEWERS COMMENTS

$ Guideline deviations are noted in Section 9.

$ There was one amendment to the Study Protocol. The actual experimental start and termination dates were changed from March 12, 2004 and March 19, 2004 to April 5, 2004 and April 12, 2000. The study was repeated due to the unacceptable analytical results of the previous definitive study trial.

Sign-off Date : 11/04/05
DP Barcode No. : D305528